Synthesis of Multiple Peptides on Plastic Pins

Scanning protein sequences by bioassay for smaller bioactive peptide sequences requires a source of many peptides homologous with the parent protein sequence. This unit deals with one of the synthetic methods for making such sets of peptides (see Fig. 18.2.1). The key to preparing large numbers (hundreds to thousands) of synthetic peptides in a short time and at minimal cost is to use a parallel synthesis technique which is efficient and can be done on a small scale. The multipin technology is suitable because it can be performed without expensive synthesizers and it uses equipment available to most laboratories. Prior experience with organic synthesis techniques or peptide chemistry is useful but not essential. The products of synthesis by multipin technology are unpurified peptides which are useful as screening reagents and may also be used to prepare purified peptide on a small scale.

Most multipin techniques exploit the conventional 8 × 12 matrix layout of common microtiter equipment which simplifies handling of the synthesis, the products (peptides), and the test results. Computer assistance with synthesis and data analysis also speeds the cycle from designing the experiment through analyzing the results.

With multipin technology, peptides are synthesized in parallel on plastic "pins" (Fig. 18.2.2) to give sets of peptides suitable not only for B and T cell epitope scanning but also for other bioassays. Peptides can be either permanently bound to the surface of the plastic for direct binding assays, or they can be released into solution. There is a choice of N- and C-terminal peptide endings. For solution-phase peptides, the synthesis scale can be 1 or 5 μmol (for a 10-mer, ~1 mg or 5 mg, respectively). The preferred coupling/deprotection chemistry used is the milder 9-fluorenylmethyloxycarbonyl (Fmoc) protection scheme rather than the older *t*-butyloxycarbonyl (*t*-Boc) protection scheme (see *UNIT 18.1*), thus reducing the level of chemical safety risk arising from synthetic peptide chemistry.

This unit covers the strategy of the multiple peptide approach to biological scanning, the synthetic protocols, and the handling of peptides after synthesis—cleavage, preliminary purification, storage, and analysis (see Basic Protocol). It is specific for the multipin technique using equipment obtained from Chiron Technologies, although some of the approaches are applicable to other multiple synthesis techniques. Procedures for multipin equipment obtained from other suppliers may differ from the procedures described here, and the manufacturer's literature should be consulted. This unit also includes protocols for preparing Fmoc–amino acid solutions (see Support Protocol 1) and for acetylating (see Support Protocol 2) or biotinylating (see Support Protocol 3) synthesized peptides.

STRATEGIC PLANNING

For a protein whose primary structure is known, the conceptually simplest method of locating all the bioactive linear peptide sequences is to make all possible peptide subsets of the protein sequence and test them. If only selected parts of the sequence are synthesized, or only the predicted active parts, bioactive sequences could be missed. The use of a set of highly overlapping peptides likewise reduces the possibility that the most bioactive sequences might be missed because they are absent from the set. A set of all overlapping 20-mers offset along the sequence by one residue at a time should capture the entire set of, for example, helper T cell epitopes, and this is a much more reliable approach than trying to predict motifs. In reality, a synthetic peptide scan through a protein is a compromise between the cost and effort in making and screening all peptides and the

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need for completeness. Thus, one worker may choose to make all overlapping 8-mers to find the linear (continuous) B cell epitopes, and another may make 12-mers offset along the sequence by five residues for the same purpose. In each case, all sequences of eight residues from the protein are present in at least one peptide, but the latter approach requires only one-fifth the number of peptides.

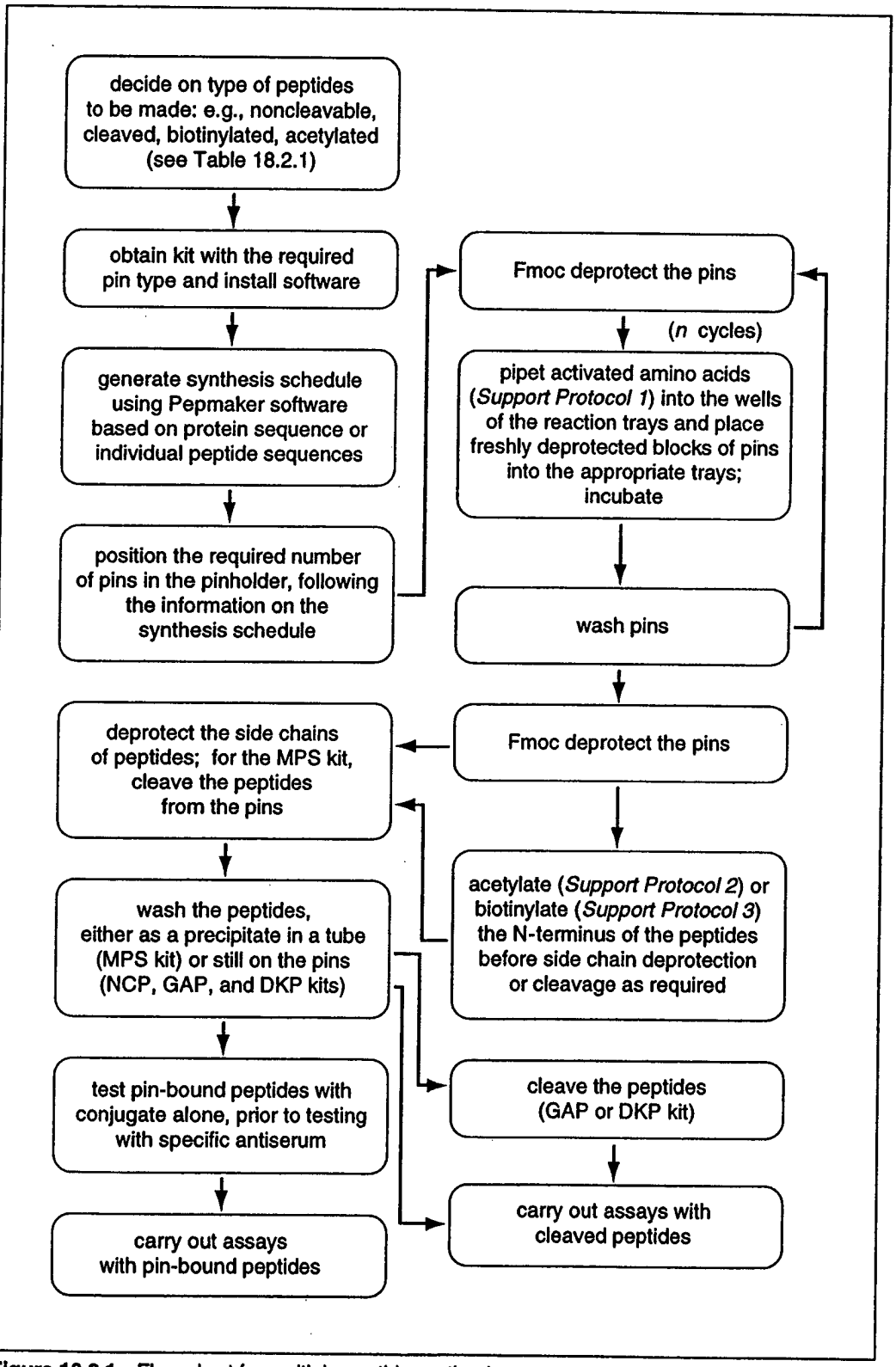

Figure 18.2.1 Flow chart for multipin peptide synthesis.

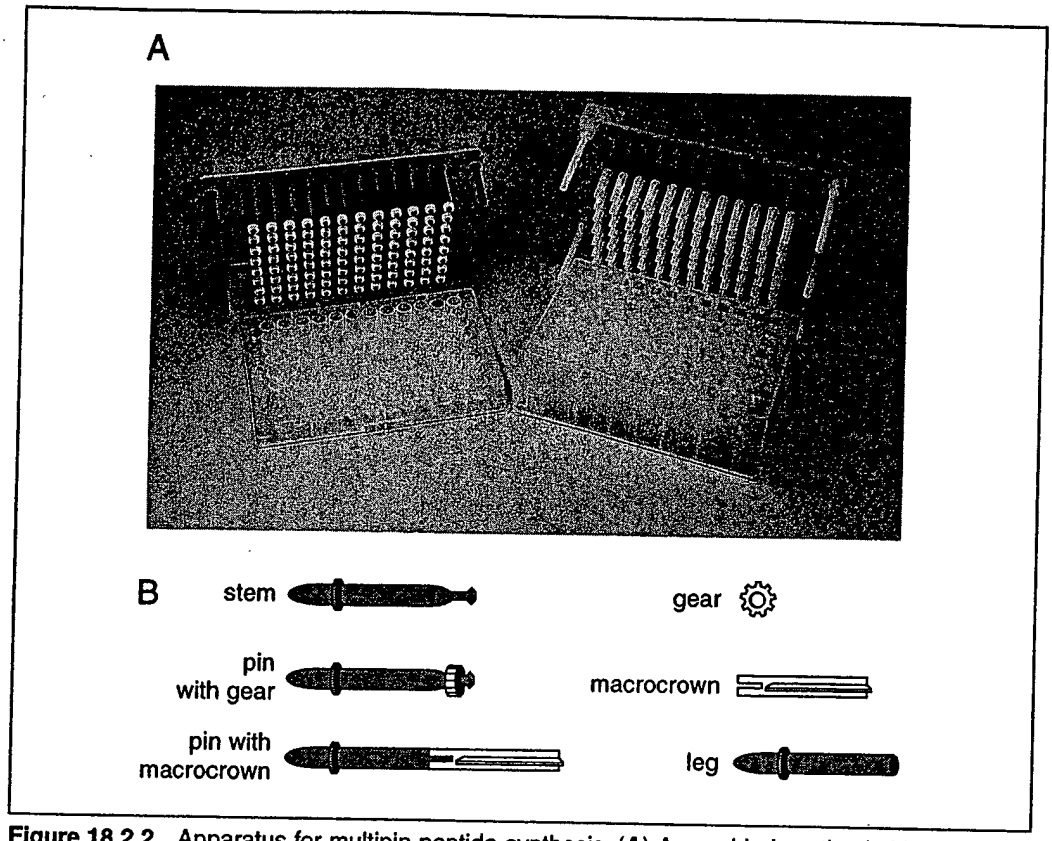

Figure 18.2.2 Apparatus for multipin peptide synthesis. (**A**) Assembled synthesis block with 96 gears (left) or 96 macrocrowns (right). (**B**) Components of the pin assembly. Components are either push-fitted together (e.g., legs or stems into the pin holder) or clipped on (gears or macrocrowns onto stems). All components are solvent-resistant plastic, either polyethylene, polypropylene, or copolymers of these two monomer types.

Planning the Synthesis

Synthetic peptides are assembled by solid-phase synthesis one amino acid at a time, commencing with the C-terminal end of the peptide on the solid phase (see *UNIT 18.1*).

The assembly process, or coupling, requires activation of the α-carboxyl group of each incoming amino acid to make it reactive with the α-amino group of the growing peptide chain. To prevent unwanted polymerization or side reaction, reactive groups in each amino acid must be temporarily protected, and the protecting group removed before further reaction can be carried out. The protecting group on the α-amino function of the most recently added amino acid must be removed before another amino acid can be coupled to it, so the α-amino protection must be labile under conditions that do not remove side-chain protection. Later, the side-chain-protecting groups must be removable under conditions that do not attack the peptide bonds. The two common protecting group "schemes" are known as *t*-butoxycarbonyl (*t*-Boc) or 9-fluorenylmethyloxycarbonyl (Fmoc). The protecting group scheme currently recommended for multipin peptide synthesis is the milder Fmoc scheme, which is the only scheme described in this chapter.

Before beginning to plan the actual synthesis in detail, a choice needs to be made regarding how the peptides will eventually be presented in the bioassay. The options available to investigators are listed in Table 18.2.1.

For noncleavable peptide (NCP) kits, peptides are permanently bound on the solid phase (pin surface) and can be used for direct binding assays but not for interaction with living cells or other complex (e.g., multicomponent) systems. In this case, the peptides must be

Table 18.2.1 Types of Pins for Multipin Peptide Synthesis[a]

Name	Linker[b]	Physical format[c]	Loading	Final form of peptide
NCP	Noncleavable	Gear	50 nmol	(N-capping)-PEPTIDE-linker-pin
MPS	AA ester	Macrocrown	5 μmol	(N-capping)-PEPTIDE-acid
MPS	Rink amide	Macrocrown	5 μmol	(N-capping)-PEPTIDE-amide
DKP	DKP-forming	Gear	1 μmol	(N-capping)-PEPTIDE-DKP
GAP	Glycine ester	Gear	1 μmol	(N-capping)-PEPTIDE-glycine-acid

[a]Abbreviations: DKP, diketopiperazine; GAP, glycine acid peptide; MPS, multiple peptide synthesis; NCP, noncleavable peptide; (N-capping), a free amine, acetyl group, or biotin; PEPTIDE, the sequence of the peptide being made.

[b]Nature of linker between peptide and graft polymer on the pin: noncleavable linker, β-alanine-hexamethylenediamine; DKP, diketopiperazine; AA ester, amino acid ester; Rink amide, Rink amide–forming linker.

[c]See Figure 18.2.2B.

"regenerated" between repeat assays by disrupting the peptide-ligand interaction without damaging the peptide. The quantity of peptide made is very small (50 nmol), but it is sufficient to provide a high surface density of peptide for direct binding assays.

In the other options, peptides are synthesized on pins and then released into solution. The mechanism of peptide release into solution affects the postsynthesis handling and thus the suitability of peptides produced by each cleavage method for various assay systems.

For multiple peptide synthesis (MPS) kits, the released peptides have a "native" free acid or an amide carboxy terminus. To make free acid C-termini, it is necessary to use macrocrowns that already have the first (C-terminal) amino acid on them because the chemistry of forming the first (ester) link is too difficult for the inexperienced user. In contrast, the Rink amide linker allows formation of a peptide with a C-terminal amide of any amino acid by adding the C-terminal amino acid to the Rink handle macrocrown using the standard amino acid coupling protocol. A Rink amide linker is a linker that can accept an amino acid but then can be cleaved in trifluoroacetic acid (TFA) to release the amide form of that amino acid (Rink, 1987). Although acid or amine endings are often the most desirable peptide format to have, they are also the most complex to produce because the cleavage of the peptides from the pin is into neat TFA plus scavengers which needs to be evaporated to recover the peptide. The scale of peptide synthesis for MPS kits is 5 μmol (~5 mg of a decamer).

For glycine acid peptide (GAP) kits, peptides with a glycine at the carboxy terminus are cleaved as the free acid, so that the C-terminal residue is a natural amino acid (glycine) and is not blocked. The peptides are also relatively simple to release from the pin and require little postsynthesis handling. However, the presence of glycine at the C-terminus may be undesirable where the C-terminus plays an important role in peptide bioactivity. The scale of synthesis for GAP kits is 1 μmol (~1 mg of a decamer).

In diketopiperazine (DKP) kits, peptides are synthesized with a DKP group at the C terminus. The DKP group is a cyclic dipeptide formed from C-terminal lysine and proline residues during the facile cleavage of the peptide under the mildest possible conditions: neutral aqueous buffer. In applications where the presence of the DKP group is acceptable, this type of peptide can make the downstream processing of synthetic peptides very simple and fast. The peptides can be placed into a bioassay system immediately after completing the cleavage. The scale of synthesis for DKP kits is 1 μmol (~1 mg of a decamer).

For these five kit options, it is also possible to choose a variety of N-terminal endings on the peptides. For example, it may be desirable to acetylate pin-bound peptides (see Support Protocol 2) to eliminate the positive charge that would otherwise be present on

the α-amino group of the N-terminal residue, or to enhance the activity of a peptide in a T helper assay (Mutch et al., 1991). A handy option for cleaved peptides is to place a biotin group on the N-terminus (see Support Protocol 3) so the peptide can be captured using avidin or streptavidin. These additions must be made prior to side-chain deprotection of the peptides.

There are other configurations for multiple peptide synthesis—e.g., the SPOTS or "peptides on paper" system (Zeneca/CRB), the RaMPS system (DuPont), and multi-synthesizer machines (e.g., Advanced ChemTech).

Assessing Peptide Sequences

Peptides differ so much in properties that it is important to assess the likely properties of the peptides before attempting to synthesize them. Peptide length and hydrophobicity are the two main attributes affecting successful synthesis. The longer the peptide, the lower will be the purity of the product, as each amino acid coupling cycle is never 100% efficient. Synthesis of peptides longer than 20 residues should be avoided unless special attention can be given to each sequence. Hydrophobic peptides may be difficult to synthesize, but more significantly they may be poorly soluble in aqueous buffers, restricting their ultimate usefulness in bioassays. Prior to beginning synthesis of a set of peptides, it is sensible to assess them all for hydrophobicity (Fauchere and Pliska, 1983; *UNIT 2.2*) and decide if all should be attempted as they stand. In many cases, it is possible to choose slightly different peptides (longer, shorter, or using a different starting and finishing point in the homologous protein sequence) that will have more user-friendly properties.

As well as these general factors affecting peptides, particular peptide sequences may have characteristics that make them difficult to synthesize, or they may be problematic after synthesis. It is not feasible to discuss all the common problems here. To help assessment of peptide sequences, a software application called Pinsoft is available free from Chiron Technologies. This allows any sequence to be typed in, and an assessment is automatically reported.

Generating Peptide Sequences

Computer software (Pepmaker) supplied with synthesis kits allows sets of overlapping peptide sequences to be generated from a protein sequence computer file using the single-letter amino acid code. Alternatively, sequences can be created using a word processor and the resulting computer text file can then be used by Pepmaker to guide synthesis. The use of this software simplifies the otherwise complex and tedious task of adding the right amino acids to each reaction plate on each synthesis cycle.

MULTIPIN SYNTHESIS OF PEPTIDES

BASIC PROTOCOL

Derivatized pins with macrocrowns or gears are attached to a pin holder. Each peptide is built up on the reactive surface of one pin by successive cycles of amino acid coupling, followed by washing and removal of the 9-fluorenylmethyloxycarbonyl (Fmoc) amino-protecting group to prepare for the next amino acid coupling cycle. A critical step is properly dispensing activated amino acid solutions into the appropriate wells of each reaction tray. A list of the well locations for dispensing of each amino acid is generated by the Pepmaker software for this purpose. When the peptides are complete, trifluoroacetic acid (TFA) that contains scavengers is used to remove the side-chain-protecting groups, and for MPS kits, to cleave the peptides from the pins. The manual provided with each type of kit (see Table 18.2.1) includes instructions and hints for kit-specific procedures.

NOTE: All reagents should be of the highest grade possible, preferably peptide synthesis or analytical reagent grade.

Materials

20% (v/v) piperidine/dimethylformamide (DMF; see recipe)
DMF, analytical reagent grade
Methanol, analytical reagent grade
100 mM activated 9-fluorenylmethyloxycarbonyl (Fmoc)–protected amino acid solutions (see Support Protocol 1)
Side chain deprotecting (SCD) solution (see recipe)
Acidified methanol: 0.5% (v/v) glacial acetic acid/methanol
1:2:0.003 (v/v/v) ether/petroleum ether/2-mercaptoethanol (2-ME)
1:2 (v/v) ether/petroleum ether
0.1 M NaOH
0.1 M acetic acid
0.1 M sodium phosphate buffer, pH 8.0 (*APPENDIX 2E*)
Sonication buffer (see recipe)

Peptide Synthesis Starter Kit (e.g., Chiron Technologies) of the desired type, containing:
  Pepmaker computer program and ELISA reading and plotting programs
  Manual
  Pins with gears or macrocrowns
Storage boxes or sealable bags, polyethylene or polypropylene (ICN Biomedicals)
Pipettor tips, polyethylene or polypropylene (ICN Biomedicals)
0.3- or 1.5-ml reaction trays, polyethylene or polypropylene (Chiron Technologies, Nunc, or Beckman)
Sonicator with power output of ~500 W
Dry nitrogen
Rack containing 96 1-ml polypropylene tubes (Bio-Rad)
10-ml capped conical polypropylene centrifuge tubes

Additional reagents and equipment for N-terminal acetylation (see Support Protocol 2; optional) or biotinylation (see Support Protocol 3; optional)

CAUTION: Perform all chemistry steps in a well-functioning chemical fume hood. Wear solvent-resistant gloves, safety glasses, and protective clothing. The reagents can be flammable, toxic, and/or carcinogenic. Avoid sources of contamination which may affect the pins, including direct contact with the bench surface or exposure to vapors. The reagents for multipin synthesis can be handled in unsealed systems, but the amount of time that these reagents are left exposed to the open air should be minimized by using capped containers for liquids or polyethylene bags for pins wherever practical. Local regulations for safe disposal of solvents and used reagents must be followed.

Prepare synthesis schedule and equipment

1. Use the Pepmaker computer program according to the instructions to generate the required set of peptide sequences (Fig. 18.2.3). Generate the printouts, which show for each coupling cycle how much of each amino acid solution, catalyst, and activating agent needs to be prepared (see Fig. 18.2.4) and where each amino acid solution is to be added to the reaction tray (see Fig. 18.2.5).

   The standard microtiter plate layout is an 8 × 12 matrix, in which the eight rows are identified as A through H and the twelve columns are identified as 1 through 12. However, the Pepmaker software uses a designation in which the column number is given first followed by a number designation for the row, beginning with row H, given in parentheses—i.e., 1(1) for well H1, 1(2) for well G1, 2(1) for well H2, and 12(8) for well A12 (see Fig. 18.2.6).

2. Label each pin holder block indelibly on the top (e.g., Synthesis #1, Block A, Synthesis #1, Block B, and so forth), preferably by scratching into the plastic with a sharp tool. Place the label where it will help orient the block so that the pins are not accidentally placed into amino acid solutions in an inverted orientation. For example, keep pin H1 and well H1 at the lower left corner of the block (Fig. 18.2.6).

   Ink labels will run or disappear with exposure to solvents.

   The multipin system is based on the standard microtiter plate layout. The ***block*** *is the complete unit and consists of the* ***pin holder****, which is the support that holds 96* ***pins*** *(in an 8 × 12 array with standard ELISA microtiter plate spacing), and five* ***legs*** *to support the device and correctly position the active surfaces. A pin consists of an inert* ***stem*** *that supports either a* ***gear*** *or a* ***macrocrown****, both of which have an active surface on which the peptide is synthesized (see Figure 18.2.2). A gear is a detachable gear-shaped unit that fits on the thin end of a stem. A macrocrown is a detachable, vaned tip that fits on the thin end of a stem. It is made of high-density polyethylene and the surface is derivatized to give a solvent-compatible polymer matrix. Macrocrowns are provided in two forms: one has a linker that cleaves to give peptides with an amide at the carboxy terminus; the other has a linker that cleaves to give the free acid at the carboxy terminus and is supplied with an amino acid already attached to the linker. The* ***reaction tray*** *used for the synthesis is a polyethylene or polypropylene tray consisting of 96 wells in the standard microtiter plate 8 × 12 matrix. Shallow reaction trays (0.3-ml) are used with gears and deep trays (1.5-ml) are used with macrocrowns.*

3. Remove any pins that are not required for the first cycle of amino acid coupling and store them dry in a plastic bag in the refrigerator until needed.

   Some pins need to be removed when the peptides in the synthesis are of various lengths because the software is designed to arrange all peptides to complete their synthesis on the same (final) coupling cycle. This approach eliminates unnecessary Fmoc–deprotection cycles for pins that are designated to carry the shorter peptides. The synthesis printout from the Pepmaker software shows which pins need to be added for each cycle of amino acid addition (Fig. 18.2.5). Pins (stems) can be pushed out from the top side of the pin holder. In the case of the MPS kit, where the first amino acid is already on the macrocrown as supplied, choose and mount the correct macrocrown for each position on the block.

Deprotect α-amino groups

4. Add 20% piperidine/DMF to a bath and place the pins in the bath so that the tips (macrocrowns or gears) are covered. Cover and let stand for 20 min at room temperature.

   CAUTION: Piperidine is flammable.

   The volume of reagent needed for all the bath steps depends on the shape of the bath, the critical factor being that all surfaces of the pins (i.e., the gears or macrocrowns) bearing the peptide need to be totally covered. A small bath suitable for gears is the upturned polypropylene lid of a pipettor tip box. For macrocrowns, deeper baths or deep-well polypropylene trays as supplied with the kit can be used.

5. Remove the block from the bath, shake off the excess liquid, and then wash the pins in a DMF bath for 2 min at room temperature.

   Again, the DMF must fully cover the tips.

6. Shake off the excess DMF and immerse the block completely in a deep bath of methanol for 2 min so that all surfaces of the block are washed.

   CAUTION: Methanol is flammable and toxic.

   In a shallower bath the block can be turned over so that the pin holder part is washed as well.

```
A      GENERAL NET SYNTHESIS SCHEDULE NUMBER : 1          Page 1
 Description: Example of a scan through Sperm Whale Myoglobin
        8-mer peptides based on the sequence MBN-SW
              Peptide spacing increment is 1
     Segment  1:  146 peptides starting at residue 1
          First peptide: [VLSEGEWQ]
          Last  peptide: [YKELGYQG]
          Protein sequence: MBN-SW (153 residues)
   1: VLSEGEWQLV  LHVWAKVEAD  VAGHGQDILI  RLFKSHPETL  EKFDRFKHLK
  51: TEAEMKASED  LKKHGVTVLT  ALGAILKKKG  HHEAELKPLA  QSHATKHKIP
 101: IKYLEFISEA  IIHVLHSRHP  GNFGADAQGA  MNKALELFRK  DIAAKYKELG
 151: YQG
              Amino Acid set to be used - AASET1
   aaset 1:Free acid L-Fmoc amino acids - DIC/HOBt chemistry
 Number of copies of each peptide  1
 Schedule based on a 250 microliter fill/well
   (Well concentration is 100 mM)
```

Figure 18.2.3 *(above and at right)* A portion of the synthesis schedule worksheets generated by Pepmaker software for Schedule no. 1 for synthesis of a set of all possible overlapping octamers of sperm whale myoglobin. **(A)** This page of the synthesis schedule is a summary of the features of the protein including its sequence. **(B)** This page of the synthesis schedule shows the sequences of the first 96 peptides that will be synthesized, the first two of which are the controls, PLAQGGGG and GLAQGGGG. Peptide sequences are shown in the conventional amino-to-carboxy-terminal direction (from left to right), with a "<" sign indicating the end attached to the solid phase during synthesis. Because amino acid couplings are carried out in the carboxy-to-amino direction, the first amino acids coupled are at the right-hand end of each sequence, adjacent to the "<".

7. Place the block in a second methanol bath to fully cover the tips. Wash for 2 min. Repeat this washing step again with a fresh methanol bath for a total of three methanol washes.

8. Remove the block and allow it to air dry in an acid-free fume hood for a minimum of 30 min.

   Avoid exposure to acidic fumes as this could prevent efficient coupling in the next step.

   The block can be conveniently left to dry while the amino acid solutions are being dispensed.

Dispense activated amino acid solutions

9. Dispense the required volume of each activated amino acid solution (see Support Protocol 1; 150 µl for gears or 450 µl for macrocrowns) into the appropriate wells of the reaction tray as specified by the synthesis schedule for the coupling cycle (e.g., Fig. 18.2.5).

Perform the amino acid coupling

10. Place the pins in the activated amino acid solutions in the reaction tray, ensuring that the block is correctly oriented before actually lowering the pins into the solution. Incubate ≥2 hr at 20° to 25°C in a polyethylene box with a lid or in a sealable polyethylene bag.

    Coupling begins immediately and is irreversible.

B GENERAL NET SYNTHESIS SCHEDULE NUMBER : 1 Page 2

Amino terminus is printed on the left

1	A 1(1)PLAQGGGG<	49	A 1(5)KHLKTEAE<
2	A 2(1)GLAQGGGG<	50	A 2(5)HLKTEAEM<
3	A 3(1)VLSEGEWQ<	51	A 3(5)LKTEAEMK<
4	A 4(1)LSEGEWQL<	52	A 4(5)KTEAEMKA<
5	A 5(1)SEGEWQLV<	53	A 5(5)TEAEMKAS<
6	A 6(1)EGEWQLVL<	54	A 6(5)EAEMKASE<
7	A 7(1)GEWQLVLH<	55	A 7(5)AEMKASED<
8	A 8(1)EWQLVLHV<	56	A 8(5)EMKASEDL<
9	A 9(1)WQLVLHVW<	57	A 9(5)MKASEDLK<
10	A10(1)QLVLHVWA<	58	A10(5)KASEDLKK<
11	A11(1)LVLHVWAK<	59	A11(5)ASEDLKKH<
12	A12(1)VLHVWAKV<	60	A12(5)SEDLKKHG<
13	A 1(2)LHVWAKVE<	61	A 1(6)EDLKKHGV<
14	A 2(2)HVWAKVEA<	62	A 2(6)DLKKHGVT<
15	A 3(2)VWAKVEAD<	63	A 3(6)LKKHGVTV<
16	A 4(2)WAKVEADV<	64	A 4(6)KKHGVTVL<
17	A 5(2)AKVEADVA<	65	A 5(6)KHGVTVLT<
18	A 6(2)KVEADVAG<	66	A 6(6)HGVTVLTA<
19	A 7(2)VEADVAGH<	67	A 7(6)GVTVLTAL<
20	A 8(2)EADVAGHG<	68	A 8(6)VTVLTALG<
21	A 9(2)ADVAGHGQ<	69	A 9(6)TVLTALGA<
22	A10(2)DVAGHGQD<	70	A10(6)VLTALGAI<
23	A11(2)VAGHGQDI<	71	A11(6)LTALGAIL<
24	A12(2)AGHGQDIL<	72	A12(6)TALGAILK<
25	A 1(3)GHGQDILI<	73	A 1(7)ALGAILKK<
26	A 2(3)HGQDILIR<	74	A 2(7)LGAILKKK<
27	A 3(3)GQDILIRL<	75	A 3(7)GAILKKKG<
28	A 4(3)QDILIRLF<	76	A 4(7)AILKKKGH<
29	A 5(3)DILIRLFK<	77	A 5(7)ILKKKGHH<
30	A 6(3)ILIRLFKS<	78	A 6(7)LKKKGHHE<
31	A 7(3)LIRLFKSH<	79	A 7(7)KKKGHHEA<
32	A 8(3)IRLFKSHP<	80	A 8(7)KKGHHEAE<
33	A 9(3)RLFKSHPE<	81	A 9(7)KGHHEAEL<
34	A10(3)LFKSHPET<	82	A10(7)GHHEAELK<
35	A11(3)FKSHPETL<	83	A11(7)HHEAELKP<
36	A12(3)KSHPETLE<	84	A12(7)HEAELKPL<
37	A 1(4)SHPETLEK<	85	A 1(8)EAELKPLA<
38	A 2(4)HPETLEKF<	86	A 2(8)AELKPLAQ<
39	A 3(4)PETLEKFD<	87	A 3(8)ELKPLAQS<
40	A 4(4)ETLEKFDR<	88	A 4(8)LKPLAQSH<
41	A 5(4)TLEKFDRF<	89	A 5(8)KPLAQSHA<
42	A 6(4)LEKFDRFK<	90	A 6(8)PLAQSHAT<
43	A 7(4)EKFDRFKH<	91	A 7(8)LAQSHATK<
44	A 8(4)KFDRFKHL<	92	A 8(8)AQSHATKH<
45	A 9(4)FDRFKHLK<	93	A 9(8)QSHATKHK<
46	A10(4)DRFKHLKT<	94	A10(8)SHATKHKI<
47	A11(4)RFKHLKTE<	95	A11(8)HATKHKIP<
48	A12(4)FKHLKTEA<	96	A12(8)ATKHKIPI<

Figure 18.2.3 *(continued)*

Wash the pins

11. Remove the block of pins from the amino acid solutions and, if the next cycle is to start immediately, place the block in a methanol bath in which the pins are immersed to half their height (tips are fully immersed). Wash with agitation for 5 min. Flick off excess methanol and allow to air dry for 2 min.
12. Place the block in a DMF bath in which the pins are immersed to half their height (tips are fully immersed). Wash with agitation for 5 min.

    If extra pins are to be added as synthesis progresses (if the peptides being made differ in length), add the pins to the appropriate spaces (see Fig. 18.2.5).

13. Repeat steps 4 through 12 for each cycle of amino acid addition. Follow the synthesis schedule for preparing Fmoc–protected amino acid solutions and for depositing

GENERAL NET SYNTHESIS SCHEDULE NUMBER : 1 Page 3
Bulk solutions for activator and/or additives (150 wells)
Chemistry Group 1 data for synthesis coupling 1
Activator : DIC requires 567.9 mg in 9.0 ml of DMF
Additive 1: HOBt requires 816.4 mg in 35.5 ml of DMF

WEIGHTS FOR INDIVIDUAL AMINO ACID SOLUTIONS

AA	#	Amino acid description	Batch	Weight (mg) Target	Actual	DIC (ml)	HOBt (ml)
A	17	Fmoc-L-Ala-OH.H20	.....	155.2	.....	0.94	3.77
D	6	Fmoc-L-Asp(OtBu)-OH	.....	75.1	.....	0.36	1.46
E	12	Fmoc-L-Glu(OtBu)-OH.H2O	.....	150.8	.....	0.68	2.72
F	6	Fmoc-L-Phe-OH	.....	70.7	.....	0.36	1.46
G	14	Fmoc-Gly-OH	.....	116.7	.....	0.79	2.30
H	12	Fmoc-L-His(Boc)-OH.5DCM	.....	176.8	.....	0.68	2.72
I	9	Fmoc-L-Ile-OH	.....	92.3	.....	0.52	2.09
K	19	Fmoc-L-Lys(Boc)-OH	.....	245.4	.....	1.05	4.19
L	17	Fmoc-L-Leu-OH	.....	166.5	.....	0.94	3.77
M	2	Fmoc-L-Met-OH	.....	28.8	.....	0.16	0.62
N	2	Fmoc-L-Asn(Trt)-OH	.....	46.2	.....	0.16	0.62
P	4	Fmoc-L-Pro-OH	.....	43.9	.....	0.26	1.04
Q	5	Fmoc-L-Gln(trt)-OH	.....	95.4	.....	0.31	1.25
R	4	Fmoc-L-Arg(PMC)-OH.3IPE	.....	90.4	.....	0.26	1.04
S	5	Fmoc-L-Ser(tBu)-OH	.....	59.9	.....	0.31	1.25
T	5	Fmoc-L-Thr(tBu)-OH	.....	62.1	.....	0.31	1.25
V	7	Fmoc-L-Val-OH	.....	70.8	.....	0.42	1.67
W	1	Fmoc-L-Trp(Boc)-OH	.....	27.0	.....	0.10	0.41
Y	3	Fmoc-L-Tyr(tBu)-OH	.....	47.7	.....	0.21	0.83

Figure 18.2.4 This page of the synthesis schedule is used for the preparation of activated amino acid solutions. It shows the amounts of each amino acid (represented by the single letter code, A through Y, along the left-hand margin), activator (diisopropylcarbodiimide [DIC] in dimethylformamide [DMF]), and catalyst (additive; 1-hydroxybenzotriazole [HOBt] in DMF) needed for the first amino acid coupling cycle. (In this example the amounts are calculated for a 250-µl reaction volume.) The amino acid powder is dissolved in the HOBt/DMF solution (right-hand column) before activation with DIC/DMF solution.

```
GENERAL NET SYNTHESIS SCHEDULE NUMBER : 1          Page 4
        PIN POSITIONS for Synthesis coupling  1
                        NEW PIN POSITIONS
A 1(1) TO B 6(5)

                Well positions for amino acid dispensing

A  A10(1)  A 2(2)  A 5(2)  A12(4)  A 4(5)  A 6(6)  A 9(6)
     A 7(7)  A 1(8)  A 5(8)  B11(1)  B 2(3)  B 4(3)  B 7(3)
     B11(3)  B 8(4) TO B 9(4)

D  A 3(2)  A10(2)  A 3(4)  A 7(5)  B 3(3)  B 6(4)

E  A 1(2)  A 9(3)  A12(3)  A11(4)  A 1(5)  A 6(5)  A 6(7)
     A 8(7)  B 6(1)  B10(1)  B 1(4)  B 1(5)

F  A 4(3)  A 2(4)  A 5(4)  B 7(1)  B12(2)  B 3(4)

G  A 1(1) TO A 2(1) A 6(2)  A 8(2)  A12(5)  A 8(6)  A 3(7)
     B 1(1) TO B 2(1) B10(2)  B 1(3)  B 6(3)  B 3(5)  B 6(5)

H  A 7(1)  A 7(2)  A 7(3)  A 7(4)  A11(5)  A 4(7) TO A 5(7)
     A 4(8)  A 8(8)  B 2(2)  B 5(2)  B 8(2)

I  A11(2)  A 1(3)  A10(6)  A10(8)  A12(8)  B 8(1)  B12(1)
    TO B 1(2) B 7(4)

K  A11(1)  A 5(3)  A 1(4)  A 6(4)  A 9(4)  A 3(5)  A 9(5)
    TO A10(5) A12(6) TO A 2(7) A10(7)  A 7(8)  A 9(8)  B 3(1)
     B10(3)  B 5(4)  B10(4)  B12(4)

L  A 4(1)  A 6(1)  A12(2)  A 3(3)  A11(3)  A 8(4)  A 8(5)
     A 4(6)  A 7(6)  A11(6)  A 9(7)  A12(7)  B 5(1)  B 4(2)
     B12(3)  B 2(4)  B 2(5)

M  A 2(5)  B 8(3)

N  B11(2)  B 9(3)

P  A 8(3)  A11(7)  A11(8)  B 9(2)

Q  A 3(1)  A 9(2)  A 2(8)  B 5(3)  B 5(5)

R  A 2(3)  A 4(4)  B 7(2)  B 4(4)

S  A 6(3)  A 5(5)  A 3(8)  B 9(1)  B 6(2)

T  A10(3)  A10(4)  A 2(6)  A 5(6)  A 6(8)

V  A 5(1)  A 8(1)  A12(1)  A 4(2)  A 1(6)  A 3(6)  B 3(2)

W  A 9(1)

Y  B 4(1)  B11(4)  B 4(5)
```

Figure 18.2.5 This page of the synthesis schedule identifies which wells of the reaction tray receive which activated amino acid solution. Each amino acid solution is identified along the left-hand margin using the single-letter amino acid code. Individual reaction trays and pin holders (A or B in this case) are identified by letters of the alphabet, and the paired numbers—e.g., 10(1) for well 10H—identify individual wells within reaction trays according to the numbering system illustrated in Figure 18.2.6.

aliquots of the activated amino acids to the appropriate wells of the reaction tray for each cycle.

Two coupling cycles can be carried out during a normal working day, and a third coupling can be carried out overnight, so a total of three amino acids can be added to each pin during a 24-hr period.

14. Deprotect the final Fmoc amino acid by repeating steps 4 through 8. Then proceed with step 15 or step 16.

15. *Optional:* For B cell epitope scanning or for T helper cell epitope scanning, the N-terminus of the peptide can be capped by acetylation (see Support Protocol 2). To allow later recapture onto avidin, the N-terminus of the peptide can be capped with biotin or long-chain biotin (see Support Protocol 3).

Deprotect the side chains

16. Dispense the required volume of SCD solution into a bath or tubes (1.5 ml/tube) and fully immerse the peptide-bearing portion of the pins. Cover bath or cap tubes and incubate 2.5 hr at 20° to 25°C.

CAUTION: SCD solution is a toxic, corrosive liquid.

For blocks where the peptide is to remain attached to the pin during side chain deprotection (i.e., NCP, GAP, and DKP kits), side chain deprotection is carried out in a bath of reagent. For pins where the peptide is simultaneously side chain deprotected and cleaved from the pin (MPS kits), the process is carried out in individual 10-ml capped conical polypropylene centrifuge tubes that become the containers for the recovered peptide.

17a. *For NCP, GAP, or DKP kits:* Wash the pins 3 times in acidified methanol to remove the SCD solution prior to further treatment.

17b. *For MPS kit:* In a good chemical fume hood, reduce the volume of SCD solution containing cleaved peptide to ~0.1 ml either with a gentle stream of dry nitrogen gas or in a centrifugal vacuum drier (e.g., Speedvac) equipped to handle corrosive fumes. Precipitate the peptide in the remaining solution with 8 ml of 1:2:0.003 ether/petroleum ether/2-ME. Decant and discard the supernatant, and wash the

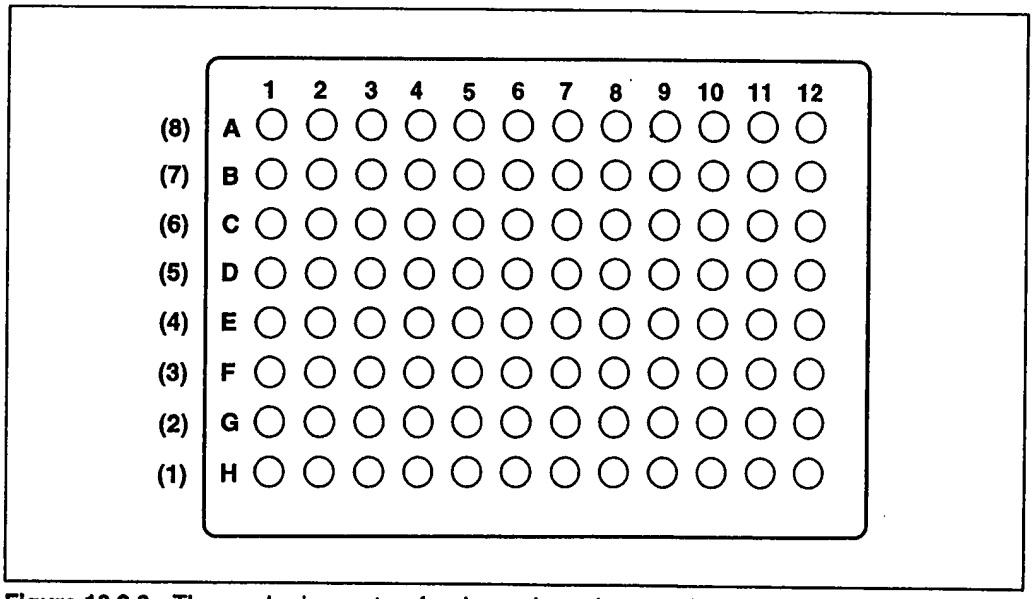

Figure 18.2.6 The numbering system for pins and reaction trays for Chiron Technologies' multipin synthesis system. Each well is identified by a pair of numbers rather than a number and a letter, e.g, 1(1). The first number identifies the column number; the second (in parentheses) identifies the lettered row, beginning with H as (1) and ending with A as (8).

precipitated peptide with 4 ml of 1:2 ether/petroleum ether. Dry the precipitate with a gentle stream of dry nitrogen.

CAUTION: Ether/petroleum ether/2-ME and ether/petroleum ether solutions are highly flammable.

These dry peptides can now be redissolved for assay purposes or may be further purified, e.g., by HPLC (see UNIT 11.6).

Prepare the peptides

18a. *For GAP kits:* Add 0.7 ml of 0.1 M NaOH to each tube of a rack of 96 1-ml polypropylene tubes. Place the pins into the solution and incubate ~1.5 hr to cleave the peptides from the pins. Immediately after cleavage, neutralize with one equivalent of 0.1 M acetic acid.

Alternatively, the 0.1 M NaOH solution can contain 40% (v/v) acetonitrile to facilitate solubilization of the more hydrophobic peptides.

The incubation time can be shorter if the tubes are sonicated during cleavage.

18b. *For DKP kits:* Cleave the peptide from the pin in a suitable, reasonably well-buffered solution with a pH >7 overnight (16 hr).

The solution for cleavage—e.g., 0.1 M sodium phosphate, pH 8, or 0.1 M HEPES, pH 8—can be chosen to be compatible with the assay for which the peptides will be used; the solution should have a buffering capacity of ~0.05 M.

Again, an organic modifier such as acetonitrile can be added to the cleavage solution to facilitate solubilization of the more hydrophobic peptides.

18c. *For NCP kits:* Prepare the pins for binding assays by floating the block, pin-side down, in sonication buffer and sonicating 10 min at ~60°C. Rinse the pins first in water, then in 20° to 45°C methanol for immediate use in an assay or air dry the pins for storage until they are used in an assay.

PREPARING ACTIVATED Fmoc–PROTECTED AMINO ACID SOLUTIONS

SUPPORT PROTOCOL 1

Activated 9-fluorenylmethyloxycarbonyl (Fmoc)–protected amino acid solutions are prepared in two steps. First, the protected amino acid is dissolved in a solution of the catalyst, 1-hydroxybenzotriazole (HOBt) in dimethylformamide (DMF). Then, just before dispensing, the amino acid is activated by adding an activating agent. The following procedure illustrates the use of diisopropylcarbodiimide (DIC) as the activating reagent. DIC is a liquid and can be measured by volume.

***Additional Materials** (also see Basic Protocol)*

9-fluorenylmethyloxycarbonyl (Fmoc)–protected amino acids with side-chain-protecting groups (Sigma, Bachem, Novachem, or Chiron Technologies), stored at 4°C
Catalyst: 1-hydroxybenzotriazole (HOBt)
Activating agent: diisopropylcarbodiimide (DIC)
Dimethylformamide (DMF), amine-free
Ethanol, analytical reagent grade
5- or 10-ml glass, polyethylene, or polypropylene bottles with inert (e.g., polythylene, Teflon) lids and liners

1. Remove the Fmoc–protected amino acids from the refrigerator and allow them to come to room temperature before weighing them out.

Warming the containers to room temperature avoids the possibility of uptake of moisture from the air onto the cold solids.

The side chains of the amino acids must also be protected during peptide synthesis: t-butyl ether is used for serine, threonine, and tyrosine; t-butyl ester is used for aspartic acid and glutamic acid; t-butoxycarbonyl (t-Boc) is used for lysine, histidine, and tryptophan; 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) is used for arginine; and trityl (Trt) is used for cysteine. If benzotriazolyl-N-oxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) or benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) activation is to be used, trityl protection should be used for asparagine and glutamine. BOP and PyBOP require greater care in handling and different protection should be used for some amino acids.

2. Weigh individual amino acids and HOBt using the quantities specified for the current synthesis cycle (e.g., see Fig. 18.2.4) into separate appropriately sized clean and dry glass, polyethylene, or polypropylene bottles. Rinse the spatula with ethanol and dry it after weighing each reagent.

   The bottle caps and inserts must be inert to any of the reagents or solvents used in making up the activated solutions (they must be Teflon, not rubber).

   Care should be taken to avoid cross-contamination of amino acids by rinsing the spatula in ethanol between weighings (the spatula must be dry before each use) and by making sure that all lids, as well as the containers, are labeled so they can be replaced on the correct bottles after weighing has been completed.

3. Measure the appropriate amount of DIC (see "Activator" as in Fig. 18.2.4).

   DIC is a liquid so it is more convenient to measure it by volume rather than by weight. Multiply the indicated weight by 1.23 (based on the density of DIC, 0.815 g/ml) as a conversion factor from the calculated weight to get the required volume of DIC in microliters.

4. Prepare HOBt and DIC solutions, by pipetting the appropriate volumes of purified (amine-free) DMF as shown on the synthesis schedule (e.g., see Fig. 18.2.4).

   Both reagents should be fully dissolved in the DMF before using them to prepare the activated amino acid solutions.

5. Add the specified volume of HOBt/DMF solution (e.g., Fig. 18.2.4, column headed "HOBt") to the individual amino acids.

   Make sure the amino acids are completely dissolved before adding the activator solution.

   Unactivated amino acid solutions may be stored a few days at 4°C.

6. Activate the individual amino acid solutions by adding the specified volume of DIC/DMF solution to each amino acid solution (e.g., Fig. 18.2.4, column headed "DIC"). Mix thoroughly and use immediately for peptide synthesis.

   Activated amino acids should be prepared immediately before use and any excess should be discarded.

SUPPORT PROTOCOL 2

N-TERMINAL ACETYLATION OF PEPTIDES

N-terminal capping of the peptides is carried out after a final 9-fluorenylmethyloxycarbonyl (Fmoc)–deprotection cycle and prior to side chain deprotection. The process is similar to coupling of amino acids, except that in the case of acetylation the active reagent can be acetic anhydride rather than acetic acid. Acetic anhydride does not require activation. If acetic anhydride is not available, simply use acetic acid as if it were an amino acid (see Support Protocol 3).

Additional Materials (also see Basic Protocol)

Acetylation solution (see recipe), prepared just before use
Pins with completed peptides (see Basic Protocol)

1. Add freshly prepared acetylation solution to appropriate bath container.
2. Immerse the pins with completed peptides in acetylation solution and incubate 90 min at room temperature.
3. Wash the pins in a methanol bath and air dry.

The pins can now be used for side chain deprotection (Basic Protocol, step 16).

N-TERMINAL BIOTINYLATION OF PEPTIDES

SUPPORT PROTOCOL 3

Biotin can also be coupled to the N-terminus of peptides after N-terminal deprotection and before side chain deprotection. The reagent is used as if it were an amino acid, using the same solvent, activating agent, and catalyst.

Additional Materials (also see Basic Protocol)

Biotin or long-chain biotin
Dimethylformamide (DMF), amine-free
Diisopropylcarbodiimide (DIC)
Pins with completed peptides (see Basic Protocol)

1. Dissolve biotin in amine-free DMF to a concentration of 125 mM.
2. Prepare a 10× solution of DIC (activating agent) by dissolving 158 mg DIC in 1 ml DMF and prepare a 10× solution of HOBt (catalyst) by dissolving 192 mg HOBt in 1 ml DMF.
3. Activate the biotin with the 10× concentrate solutions of activation agent and catalyst (80:10:10 [v/v/v]).
4. Dispense 150 µl/well (for gears) or 450 µl/well (for macrocrowns) into reaction trays.
5. Immerse the pins with completed peptides in the reaction tray and incubate ≥2 hr.
6. Wash the pins in methanol.

The pins can now be used for side chain deprotection (see Basic Protocol, step 16).

REAGENTS AND SOLUTIONS

Use Milli-Q water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2E; for suppliers, see SUPPLIERS APPENDIX.

Acetylation solution

For 200 ml:
193 ml dimethylformamide (DMF)
6 ml acetic anhydride
1 ml *N*-ethyldiisopropylamine
Prepare immediately before use and discard after use

DO NOT expose pins to acetic anhydride at any other time except during acetylation. Also, do not store acetic anhydride anywhere near where peptide synthesis is performed.

The DMF does not need to be amine-free.

20% piperidine/DMF

Prepare a 20% (v/v) solution of the best quality piperidine available in analytical reagent–grade dimethylformamide (DMF). Prepare a fresh solution for each synthesis (solution can be reused several times within a synthesis). Store at room temperature in an amber bottle containing activated molecular sieves to remove moisture.

CAUTION: This solution is highly flammable and toxic.

continued

If high-quality piperidine is not available, it may have to be treated with solid sodium hydroxide and redistilled.

DMF need not be amine-free.

Side chain deprotecting (SCD) solution

33 parts (v/v) trifluoroacetic acid
1 part (v/v) ethanedithiol
2 parts (v/v) anisole
2 parts (v/v) thioanisole
2 parts (v/v) H_2O
Prepare immediately before use and do not store or reuse

CAUTION: This solution is corrosive and extremely malodorous. Contamination of the laboratory, especially with ethanedithiol, should be avoided. Wipe the outside of ethanedithiol-contaminated equipment or containers with dilute, 0.1% aqueous hydrogen peroxide to oxidize ethanedithiol to a nonodorous compound before removing the container from the fume hood. DO NOT allow hydrogen peroxide to contact other readily oxidizable materials or reagents.

Sonication buffer

1% (w/v) SDS
0.1 M sodium phosphate buffer, pH 7.2 (*APPENDIX 2E*)
0.1% (v/v) 2-mercaptoethanol (2-ME)
Store at room temperature up to 1 week

CAUTION: Before discarding sonication buffer, destroy remaining 2-ME by adding 2 ml 30% hydrogen peroxide per liter of buffer.

COMMENTARY

Background Information

The multipin method was developed by Dr. H.M. Geysen and coworkers (Geysen et al., 1984, 1987) as a scanning method for linear antibody-defined epitopes. Eventually in the late 1980s, the method was adapted to parallel synthesis of cleaved (soluble) peptides (Maeji et al., 1990), opening the way for systematic scanning of T helper (Reece et al., 1993) and cytotoxic epitopes (Burrows et al., 1994). Initially only suitable for synthesis of short peptides (up to 10 amino acid residues), the method can now routinely produce peptides of up to 20 residues of acceptable quality for initial screening experiments (Valerio et al., 1993).

Critical Parameters

Successful peptide synthesis requires reagents of a quality appropriate to the particular step, and the careful application of those reagents. For example, the protected amino acids need to be free of reactive counterions such as dicyclohexylamine (DCHA), contaminating unprotected amino acid, isomers such as the D-amino acid, and water. Check carefully that the amino acid as supplied is EXACTLY the same as specified in the manual or on the software. Apart from quality testing each amino acid, the best assurance of quality is to buy only from reputable suppliers.

Dimethylformamide (DMF) is the primary solvent for carrying out reactions (couplings) on pins. Its low volatility and moderate polarity make it suitable for dissolving the amino acids and solvating the graft polymer/growing peptide on the pin surface. Purity is not critical for some (washing) steps, but is critical for the DMF used just before and during amino acid coupling. Presence of excessive amine in the DMF results in loss of activated amino acid because the amino acid couples to the amine rather than to the peptide on the pin. Fortunately, the pin system allows use of substantial molar excesses of incoming amino acid (typically 6- to 1000-fold), so loss of some amino acid is not disastrous. Fresh DMF of the best available grade should be used for the coupling, and it is recommended that the amine level be tested using the FDNB test (Stewart and Young, 1984).

Liberal use is made of methanol as a washing solvent. Analytical reagent grade methanol is readily available at low cost in large containers (20 or 200 liters) and is relatively easy to dispose of. It is possible to reduce the use of methanol by reusing it for washes: the last wash

bath in any series should be in fresh (pure) methanol. In the next round of washes, the former last bath is then reassigned as the second-to-last wash, the previously second-to-last bath becomes the third-to-last, and so on. For each synthesis cycle, the first wash bath in the series is the one which is discarded. The presence of methanol is undesirable during reactions on the pins, but as it evaporates readily it can be easily removed by standing the block in a moving stream of air, such as the opening of an operating chemical fume hood. Methanol will dry more rapidly and the methanol-washed pins will take up less moisture from the air if the methanol is warm (e.g., prewarmed to 45°C in a closed bottle in a water bath).

Other solvents (e.g., ether, petroleum ether, acetonitrile) should be the best available grade.

Carrying out the correct synthesis of the peptides requires that all steps are performed with a very high level of attention to detail. All cyclically repeated steps (washes and deprotections) must be performed, and the activation and dispensing of the amino acids for each coupling cycle must be carried out exactly, or the peptides made may have the incorrect sequence, may be missing an amino acid, or may be truncated. Computerized equipment is available for assisting with the accurate dispensing of amino acids to the wells in a reaction tray (e.g., "Pin-Aid," Chiron Technologies; Carter et al., 1992). The growing peptides must not be subjected to conditions that would prematurely block or deprotect the side chains (for example, from premature exposure to acetic anhydride or trifluoroacetic acid, which should be stored well away from where peptide synthesis is being performed).

As a spot test for correct completion of all the steps of synthesis, it is wise to synthesize controls on each block of 96 pins. For noncleavable peptides, these controls can be peptide sequences that can be probed with an antibody known to react with the peptide. In this case, one of the two peptides should be a negative control, such as a randomized sequence. For cleavable peptides, the quantity and quality of the controls can be monitored by the usual techniques of HPLC (*UNIT 11.6*), amino acid analysis (*UNIT 11.9*), and mass spectrometry (Chapter 16). Ultimately, proof that an assay result is a function of the particular peptide made has to rely on a confirmatory experiment carried out with more highly-characterized peptide or on analysis of a sample of the particular peptide used in the experiment.

Once peptides have been made, they need to be handled and stored carefully to prevent degradation. Noncleavable peptides (pins) should be stored dry in a refrigerator after removal of any bound protein. If stored with desiccant they should be stable for months to years. Cleaved peptides can be stored frozen or as dry powder. After a long period of storage, it is wise to reassay controls or confirm the quality of the stored peptide by analysis.

Another parameter critical to data from large numbers of peptides is to ensure that the identity of each peptide is properly tracked and that activity is not ascribed to the wrong peptide. Consistent use of the 8 × 12 microtiter plate format for synthesis, storage, assay, and use of computerized records for tracking all three processes can help avoid mistakes. Tracking and control is particularly easy if the assay data is read directly from a microtiter plate reader to a computer that is programmed with the peptide information because this method avoids manual data transcription.

Anticipated Results

For a noncleavable pin-peptide synthesis, two control peptides, one of which is reactive with a monoclonal antibody in ELISA and the other serving as a nonbinding peptide control, should show the specific binding expected based on past data. For cleaved peptides, the yield of control peptide should be in the range expected from the stated pin loading (substitution level), e.g., 1 μmol for GAP and DKP kits or 5 μmol for the MPS kit. Purity of the cleaved controls should be consistent with the results of previous batches and should be of an acceptable standard.

Testing of a systematic set of peptides in a bioassay can give data that is interpretable without recourse to additional controls, because a systematic set of peptides through a protein includes many sequences that are unlikely to be reactive sequences, i.e., they act as internal negative controls. Figure 18.2.7 shows one set of ELISA data from scanning noncleaved peptides with a monoclonal antibody. In screening for T helper cell responsiveness it is critical to include many control cultures, not only controls with no peptide added but also controls with nonstimulatory peptide. Systematic sets of peptides automatically include such controls (Reece et al., 1994).

Time Considerations

If amino acid coupling is carried out at 3 cycles/day, which can fit into a conventional working day, then it will take up to 2 weeks to make a set of 15-mers, as there is extra time required for side chain deprotection and drying down (depending on the peptide format). Although this may seem slow, the fact that hundreds or thousands of peptides can be made simultaneously means that a project requiring large numbers of peptides is completed in a very short time. Indeed, the rate-limiting step may be the time it takes to carry out the assays on the large number of peptides when they become available.

From this perspective, biotinylated peptides produced on glycine acid peptide (GAP), diketopiperazine (DKP), or multiple peptide syn-

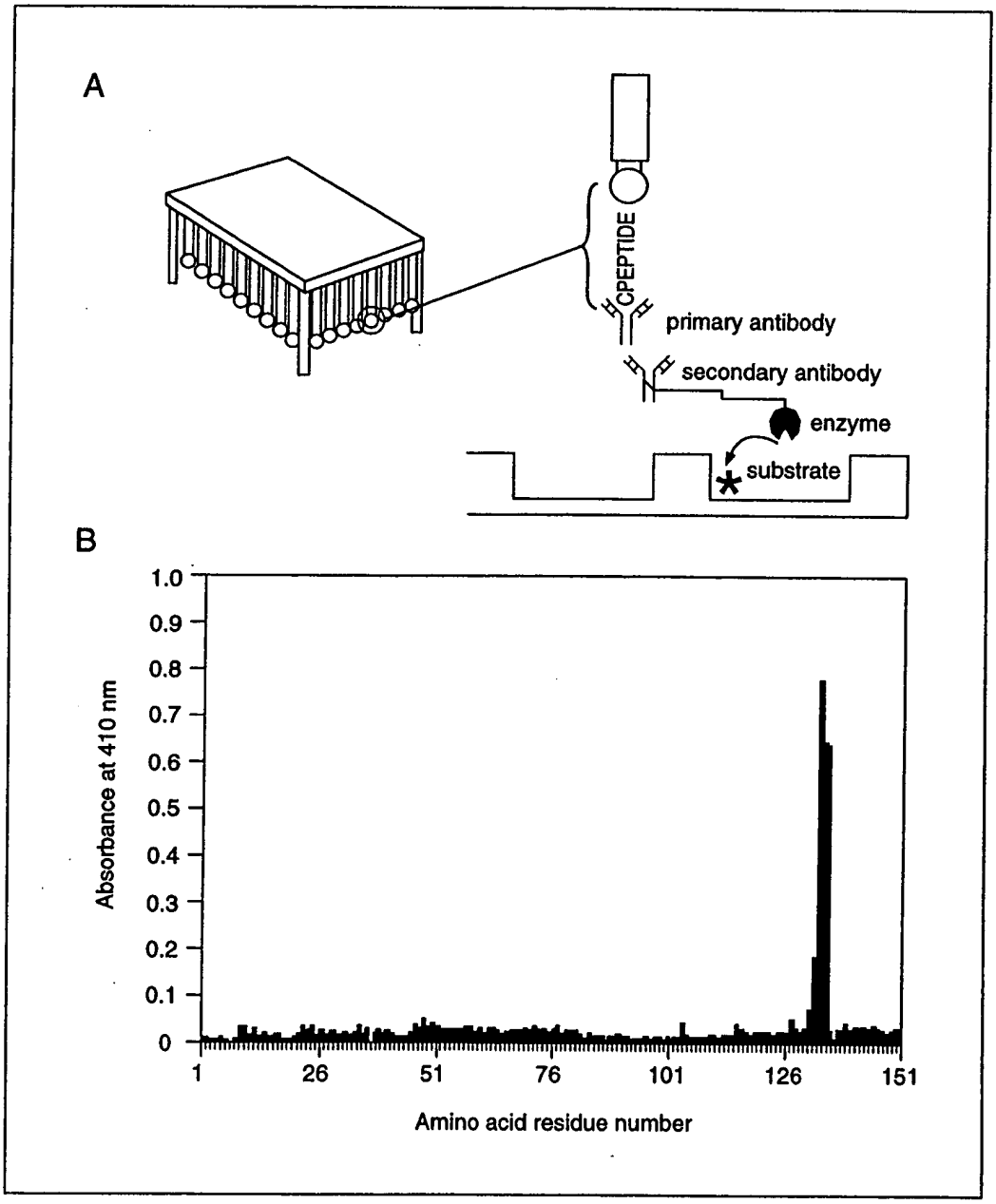

Figure 18.2.7 Multipin capture ELISA. (**A**) Setup for multipin capture ELISA. Pins (gears) with peptides covalently attached are incubated in primary antibody, secondary antibody, and substrate developer in ELISA plates. The absorbance is measured and the resulting absorbance values are graphed versus peptide number, corresponding to the N-terminal residue number of the peptide in the protein sequence. (**B**) Peptide pin capture ELISA results with a monoclonal antibody against pins bearing octamer peptides of gonococcal pilin protein. All the peptides that show high readings contain a significant portion of the epitope. (Diagram courtesy of Dr. Fred Cassels, Walter Reed Army Institute of Research, Washington, D.C.)

thesis (MPS) pins have a great advantage over the noncleavable peptide (NCP) pin-bound peptides, as the latter can only be assayed once a day, whereas hundreds of parallel assays can be carried out on all biotinylated peptides at once. Reading data directly into a computer enables the massive amounts of data to be stored efficiently for later analysis.

Dispensing amino acids can be carried out efficiently by two people, one reading out the position into which the amino acid is to be dispensed and the other doing the actual dispensing. The passive partner (reader) can also act as a cross-checker to ensure no mistakes are made. If a computer-controlled pointing device is used, accuracy is improved and dispensing becomes a one-person operation. For large syntheses (>200 peptides), it is important that the dispensing be fast and accurate so that three couplings can be carried out per day.

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